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CHANGES IN THE ULTRASTRUCTURE OF CELL WALLS, CELLULOSE SYNTHESIS, AND GLUCAN SYNTHASE ACTIVITY FROM GRAVISTIMULATED PULVINI OF OAT (AVENA SATIVA)[1]

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Ultrastructural analyses of the cell walls from top and bottom halves of gravistimulated pulvini from oat leaves show a decrease in the density of material within the cell walls from the lower halves of pulvini after 24 h of gravistimulation. Assays of cellulose synthesis with a ^{14}C-sucrose pulse-chase experiment indicate no difference in the amount of new cellulose synthesized in top compared with bottom halves of gravistimulated pulvini. The highest rate of cellulose synthesis occurs with 12–24 h of gravistimulation. Treatment of graviresponding pulvini with 2,6-dichlorobenzonitrile (DCBN) had only a minor effect on segment gravitropic curvature. We also found that there is no difference in the activities of either glucan synthase I or glucan synthase II in top halves as compared with bottom halves of gravistimulated pulvini. We conclude that the graviresponse in oat stems is not driven by new cell wall synthesis but, rather, by changes in cell wall plasticity and osmotic potential.

Introduction

The gravitropic response of the leaf-sheath pulvinus of oat is initiated by the sedimentation of starch grains and culminates in a hormone-mediated growth response (Kaufman et al. 1987; Song et al. 1988; Brock and Kaufman 1990). The growth response involves not only cell expansion but also the synthesis of new wall materials (Dayanadan et al. 1976; Brock and Kaufman 1990). Cell division is not involved in this leaf-sheath gravitropic response (Dayanadan et al. 1976).

During cell expansion in cereal grasses, a mixed linkage (1 → 3) and (1 → 4)-β-D-glucan is synthesized (Carpita 1984). The basic structure is made up of repeating units of cellotriosyl and cellotetraosyl units connected by a single (1 → 3)-β-D-glucosyl linkage (Woodward et al. 1983; Kato and Nevins 1986). It has been reported that there is relatively little difference in cellulose synthesis between top halves and bottom halves of graviresponding cereal grass pulvini (Arslan and Bennett-Clark 1958; Gibeaut et al. 1990). Dayanadan et al. (1976) reported that cells in the lower halves of graviresponding pulvini become greatly elongated. Parenchyma and collenchyma cells increase in length approximately 4.25 and 4.6 times, respectively. The elongated collenchyma cells exhibit thick and thin regions in their walls by the end of 48 h of gravistimulation. Given that there is essentially equal cellulose synthesis in top and bottom halves of gravistimulated pulvini and that the cells in the bottom half elongate greatly, we hypothesize that there should also be visible internal ultrastructural changes in top- and bottom-half cell walls of graviresponding tissue. We predict, given equal cellulose synthesis in top- and bottom-half cells, that the cell walls will most likely become less dense in the cells of the bottom halves of gravistimulated pulvini. In order to investigate these ultrastructural changes, we examined cell walls of differentially elongating cells in graviresponding oat leaf-sheath pulvini by transmission electron microscopy (TEM). We also investigated the kinetics of cellulose synthesis to determine whether cellulose biosynthesis was indeed the same in top and bottom halves throughout the graviresponse. Along with this analysis, we also investigated changes in glucan synthase activity, the putative cellulose synthase in plants (Delmer 1987).

Material and methods

Plants

Oat plants (*Avena sativa* L. cv Victory, from Svenska Allmanna A.B., Svalöf, Sweden) were grown from seed in a greenhouse with supplemental lighting to maintain a light : dark ratio of 18:6 h (Brock et al. 1989). After approximately 4 wk, stem segments were harvested for gravistimulation treatments. For experimentation, plants were selected for length of next-to-last (p-1) internode greater than 4 cm, at which stage the next-to-last (p-1) leaf-sheath pulvinus is maximally competent to show a graviresponse (Brock et al. 1989). Gravistimulation involved placing stem segments, cut 3 cm below and 5 cm above the pulvinus, horizontally between paper towels and glass plates, with 0.1 M sucrose supplied to the segment bases through the towels. Similarly prepared segments left in a vertical orientation were used as controls for comparison.

[1] Abbreviations: TEM, transmission electron microscope; GSI, glucan synthase I; GSII, glucan synthase II; UDPG, uridine 5′-diphosphate glucose; DCBN, 2,6-dichlorobenzonitrile.

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Cell wall analysis

In general, the methods of Meier et al. (1981) were followed. Briefly, oat stem segments were cut 3 cm below and just above each pulvinus with a razor blade. Segments were positioned vertically, with the lower half of each segment contacting dry filter paper, and 5 μL of uniformly labeled ^{14}C-sucrose solution (0.1 M, 10^{-4} μCi/μL) was placed on top of each cut pulvinus. Segments were left in this position for 5 min in the dark, then washed three times with cold 0.1 M sucrose solution. After washing, segments were gravistimulated as described above. After 3, 6, 9, 12, 15, 18, 21, 24, 36, and 48 h of gravistimulation, pulvinus tissue was harvested and cut into top and bottom halves (totaling approximately 1 g of tissue for top and bottom halves). Tissue was homogenized in liquid nitrogen with mortar and pestle. Unpolymerized low-molecular weight precursor components (mainly unincorporated sucrose) were extracted in boiling methanol (80%, v/v) and filtered through a GF/A glass filter (Whatman). This procedure was repeated one more time. The filtrates were combined, and radioactivity was measured with a scintillation counter. Twenty milliliters of DMSO were added to the filter residues, which were then autoclaved for 30 min and filtered through a GF/A filter disk This procedure was repeated once more; then filtrates were combined and radioactivity was measured. This fraction contained mainly callose (β-1,3-glucan). Finally, radioactivity incorporated into cellulose was assayed by measuring the radioactivity of the remaining filter residue. Each time point represents cell wall analysis of approximately 100 pulvini (about 1 g of tissue).

Glucan synthase extraction

The methods of Walton and Ray (1982) were generally followed. Four mL of grinding buffer (50 mM Tris-Cl [pH 8.0], 1 mM EDTA, 0.25 M sucrose, and 0.1 mM $MgCl_2$) were added to 1 g of pulvinus tissue, and the tissue was homogenized in liquid nitrogen using a mortar and pestle. The homogenate was centrifuged at 10,000 *g* for 10 min at 4 C. The supernatant was collected, then centrifuged at 100,000 *g* for 30 min. The pellet was resuspended in 500 μL of grinding buffer. This crude membrane preparation was used to assay glucan synthase activity.

Glucan synthase I (GSI) enzyme assay

The GSI assay procedure used was that of Walton and Ray (1982). The glucan synthase assay solution consisted of 8.0 μL of 31 mM Tris (pH 8.0), 31 mM $MgCl_2$, 0.37 μM uridine 5′-diphosphate ^{14}C-glucose (UDPG) (0.1 mCi/mmol), and 50 μL of membrane preparation. The solution was incubated at 25 C for 20 min, then 150 μL of boiled crude membrane (0.5 g fresh weight equivalent/mL) was added, to facilitate precipitation of product, and the solution was boiled for 1 min. Four mL of 70% (v/v) ethanol was added, and the solution was stored overnight at −20 C. Reaction mixtures were filtered through Whatman GF/A filter disks. Filter disks were washed with 10 mL of 70% ethanol and radioactivity measured. GSI activity was defined as the incorporation of ^{14}C-glucose in the ethanol-insoluble product. From preliminary experiments, we determined that these assay conditions produced a linear relationship of glucan production over time. GSI activity was measured in counts per minute (c.p.m.) of ^{14}C-glucose incorporated into ethanol-insoluble product during 20 min of incubation of approximately 100 mg fresh weight of pulvinus tissue. The enzyme assay was repeated three times for each time point.

Glucan synthase II (GSII) enzyme assay

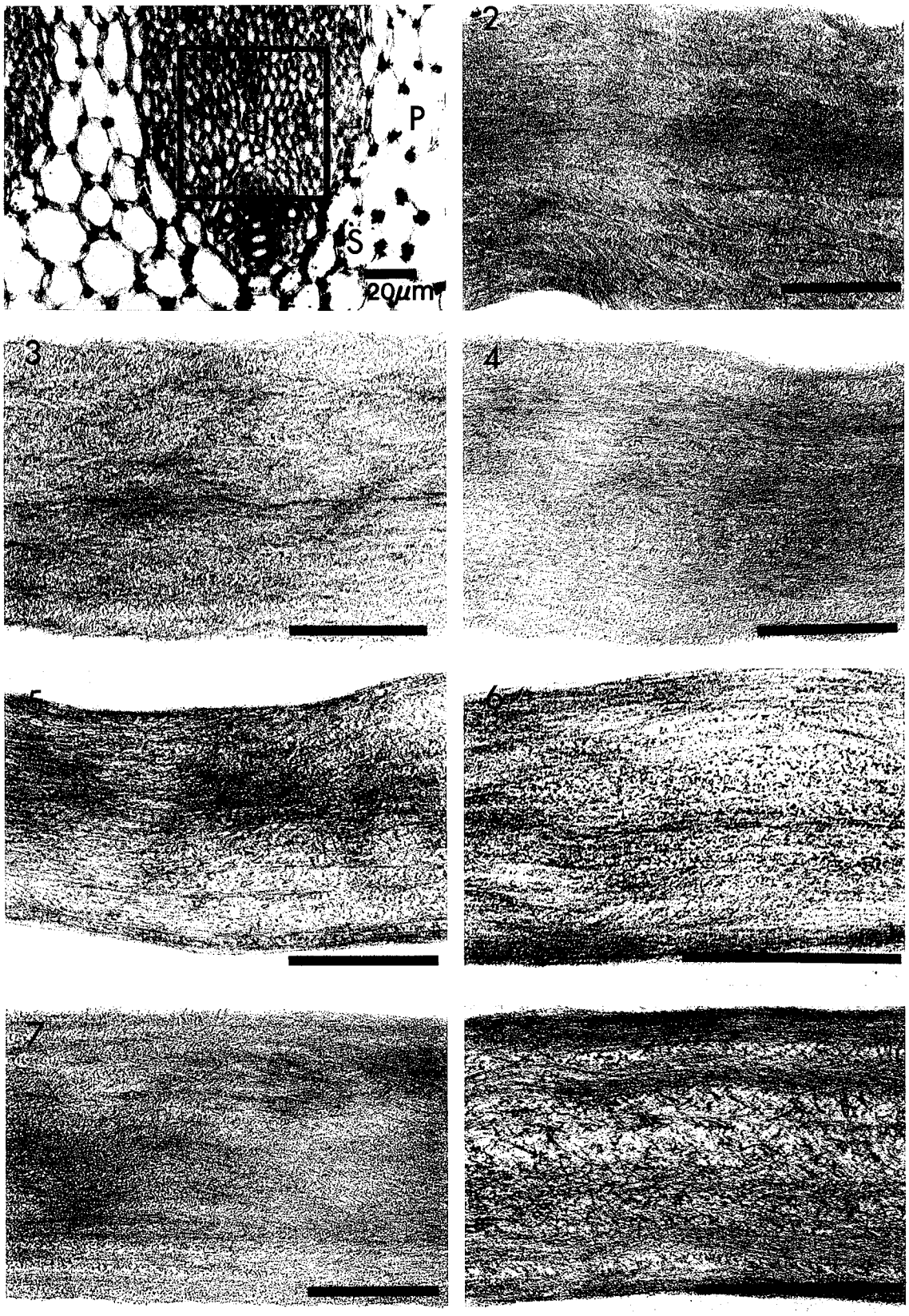
The enzyme assay for GSII was the same as for GSI except that the exogenous Mg^{2+} was replaced with $CaCl_2$, and 0.3 mM unlabeled UDPG was added in addition to the labeled UDPG. Different substrate concentrations and cation requirements enable one to assay for GSI or GSII (Ray 1979). GSII activity was defined as incorporation of ^{14}C-glucose in ethanol-insoluble product. GSII activity was measured in c.p.m. of ^{14}C-glucose incorporated into ethanol-insoluble product during 20 min of incubation of approximately 100 mg of pulvinus tissue (fresh weight). The enzyme assay was repeated three times for each time point.

Effect of DCBN on the graviresponse

The epidermis of 30 pulvini was abraded with wet carborundum by rubbing three times between the thumb and forefinger. A 1% agar block containing 0.1 M sucrose and 0.1 mM 2,6-dichlorobenzonitrile (DCBN) was applied to either the top or bottom half of each pulvinus. Segments were gravistimulated with the agar block in place for 24 h; then the curvature was measured with a protractor. This experiment was repeated two times.

Electron microscopy

Stem segments containing p-1 pulvini were gravistimulated as described above for 3, 6, 12, and 24 h and, along with 0 h control pulvini, were prepared for TEM. At each time interval, three to four different pulvini were cut transversely through the center of the pulvinus and fixed in 2% formaldehyde in 50 mM sodium phosphate buffer (pH 7.0) for 30 min at 4 C. During formaldehyde fixation, the cut pulvini were kept in the same orientation they had been in during gravistimulation. The tissue was washed



Figs. 1–8 Collenchyma cell walls from top halves and bottom halves of gravistimulated oat pulvini. Fig. 1, Light micrograph showing collenchyma tissue (*C*), vascular bundle (*V*), parenchyma (*P*), and statenchyma (*S*) containing sedimented statoliths. Gravity vector runs from right to left. Fig. 2, Zero hour collenchyma cell wall from vertical (nongravistimulated) pulvinus. Figs. 3, 5, 7, Collenchyma cell walls from top halves of gravistimulated pulvini. Figs. 4, 6, 8, Collenchyma cell walls from lower halves of gravistimulated pulvini. Figs. 3, 4, 3 h of gravistimulation. Figs. 5, 6, 12 h of gravistimulation. Figs. 7, 8, 24 h of gravistimulation. Bars on electron micrographs equal 0.5 μm.

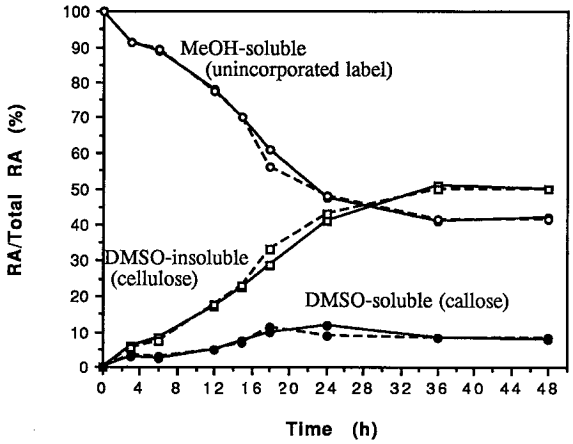


Fig. 9 Pulse-chase experiment measuring the incorporation of ^{14}C-glucose into cell walls during gravitropic response in oat pulvini. Solid line: top halves of pulvini; dotted line: bottom halves of pulvini. Open circles: MeOH-soluble fraction (unincorporated radioactive sucrose); closed circles: DMSO-soluble fraction (callose); open squares: DMSO-insoluble fraction (cellulose); *RA*: radioactivity. Each time point represents data collected from an assay of approximately 100 pulvini, ca. 1 g of tissue fresh weight.

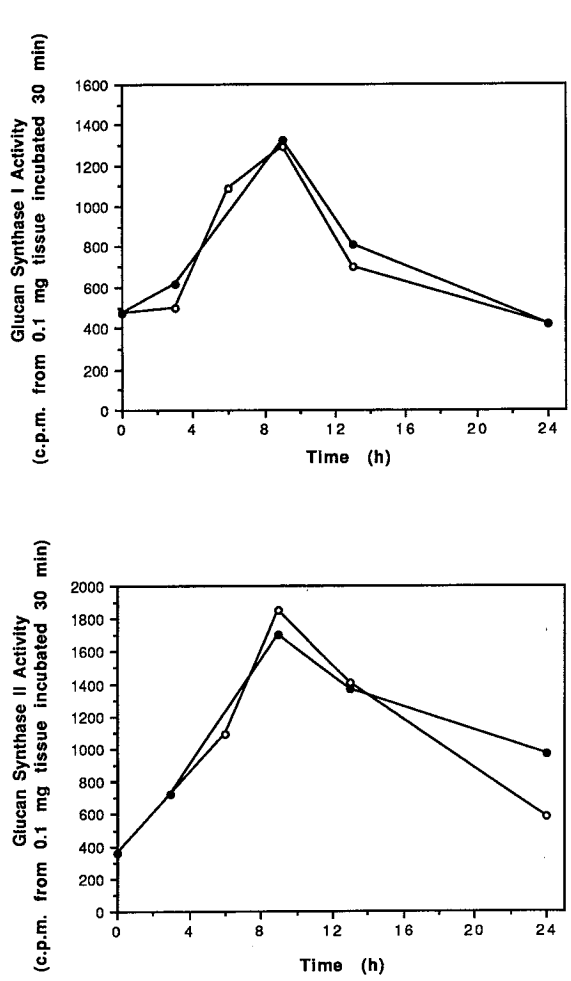


Fig. 10 Changes in glucan synthase I and II activities. *A*, Changes in glucan synthase I activity in top and bottom halves of oat pulvini during gravistimulation. *B*, Changes in glucan synthase II activity in top and bottom halves of oat pulvini during gravistimulation. Open circles: top halves of pulvini; closed circles: bottom halves of pulvini. Each point is the average of three replicate assays.

three times in 50 mM phosphate buffer, then fixed overnight in 3% glutaraldehyde in 50 mM phosphate buffer at 4 C. The tissue was washed three times in 50 mM phosphate buffer (pH 7.0) and postfixed in 2% osmium tetroxide in 50 mM phosphate buffer (pH 7.0) for 2 h at room temperature, then dehydrated in a graded series of ethanol. Sections were placed in Spurr's resin (Spurr 1969) at 30% (1 h), 50% (1 h), and 100% (overnight), then embedded in 100% Spurr's resin. The resin was allowed to polymerize at 60 C overnight. Specimens were sectioned with a diamond knife, stained with uranyl acetate followed by lead citrate, and observed in a Zeiss EM 10-CA TEM at 60 kV. Specimens from at least two different pulvini were sectioned and examined in the TEM. Micrographs are representative of cell walls found in pulvini from the various time points and pulvinus halves.

Results

Ultrastructural changes in gravireponding cell walls

Cell walls from pulvini that had been gravistimulated for 0, 3, 12, and 24 h were examined for evidence of ultrastructural changes (figs. 1–8). At 0 h, and after 3 h of gravistimulation, cell walls of the pulvini appeared to have relatively uniform densities in top and bottom halves (figs. 2, 3, 4). By 12 h of gravistimulation a difference in wall density began to become apparent, especially in the collenchyma cell walls from bottom halves of pulvini (figs. 5, 6). After 24 h of gravistimulation, obvious changes had occurred in bottom-half collenchyma cell walls; they were less dense toward the central portions of the walls when compared with top-half cell walls (figs. 7, 8).

Incorporation of ^{14}C-glucose into cellulose

In the pulse-chase experiment with ^{14}C-sucrose, we found that radioactivity was incorporated into cellulose of gravireponding pulvini during the graviresponse, and that there was no difference in the amount of radioactivity incorporated between the top and the bottom halves of gravireponding pulvini (fig. 9). There was a slight increase in radioactivity of callose in both top and bottom halves over the 48-h period of gravistimulation. There appears to be a lag phase with minimal cellulose synthesis from 0 to 6 h

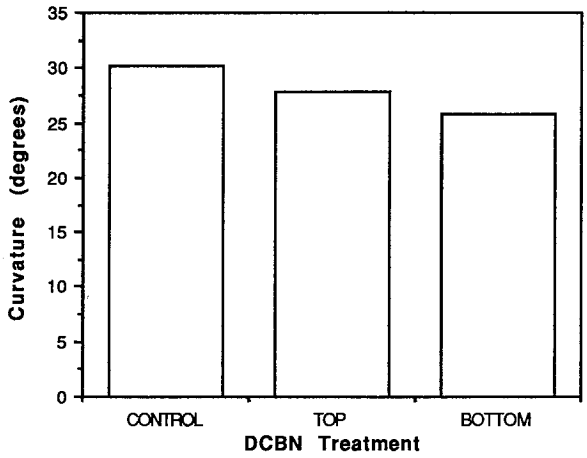


Fig. 11 Curvature produced by pulvini treated on their top or bottom half with DCBN for 24 h.

followed by an almost linear increase in the amount of cellulose synthesis from 12 to 24 h, followed by a decreasing amount of cellulose synthesis after 24 h.

Glucan synthase activity in gravistimulated pulvini

Glucan synthases I and II both showed nearly linear increases in activity during the first 8–9 h of gravistimulation; then the activity of both enzymes declined to vertical control levels by 24 h (fig. 10*A, B*). There was no significant difference in amount of glucan synthase I or of glucan synthase II activity between top and bottom halves over the entire gravistimulation period.

Effect of DCBN on the graviresponse

Segments treated with DCBN on the top half of their pulvini graviresponded to 92% of the curvature relative to the control pulvini (fig. 11). Segments treated with DCBN on the bottom half of their pulvini graviresponded to 87% of the curvature relative to the control pulvini.

Discussion

Our TEM study showed that cell walls from the lower halves of the graviresponding oat leaf-sheath pulvini, as predicted, do become less dense than cell walls from the top halves of graviresponding pulvini. This was especially evident in the collenchyma cell walls. Because cells in the lower half of a gravistimulated pulvinus greatly elongate as compared with those in the upper half, which do not, and because there is not an asymmetric increase in the synthesis of new cellulose between the lower and upper halves, the cell walls in the lower halves must become less dense. Dayanadan et al. (1976) showed that the walls of collenchyma cells in the lower halves of gravistimulated pulvini become greatly elongated and develop alternating thick and thin regions when compared with cell walls from nongravistimulated pulvini. Our analysis of cell wall ultrastructure adds to these data of physical change by showing that the cell walls in the lower half of the graviresponding pulvini also become less dense upon gravistimulation. In other words, the collenchyma cells from the lower half of gravistimulated pulvini become greatly elongated with alternating regions of thick and thin walls and show an internal decrease in cell wall density.

We found that cellulose synthesis was equal in top and bottom halves of graviresponding pulvini through 48 h of gravistimulation. The period of maximum cellulose synthesis (12–24 h) corresponds to the period of declining bending rate (after 12 h) in graviresponding pulvini (Brock et al. 1989). The finding of equal cellulose synthesis in top and bottom pulvinus halves led us to question one of our earlier results regarding glucan synthase activity (Gibeaut et al. 1990). In that study we reported finding significantly more glucan synthase activity in bottom halves of graviresponding pulvini than in top halves. After determining that cellulose synthesis is essentially equal in top and bottom halves of graviresponding pulvini, we realized that glucan synthase activity should also be equal in top and bottom halves, if glucan synthase does indeed produce cellulose in higher plants. Therefore, we reexamined our results and methods used during our initial glucan synthase assays and found that we were using an old assay technique. In addition, we had only assayed changes in glucan synthase activity at one time point, 24 h of gravistimulation.

The two glucan synthases from higher plants are characterized by having different biochemical assay requirements for substrate concentration, and they have different cation requirements (Ray 1979). Glucan synthase I activity can be measured at low UDPG concentrations (μM) in the presence of Mg^{2+}, while glucan synthase II activity requires higher substrate concentrations (mM) and the presence of Ca^{2+}. The original methods that we used for assaying glucan synthase activity included mM concentrations of UDPG combined with Mg^{2+} ions. Therefore, that particular glucan synthase assay was invalid. We believe that our present results correctly reflect glucan synthase activities in the graviresponding leaf-sheath pulvinus. These findings indicate an increased activity of glucan synthases I and II upon gravistimulation (fig. 10) and a pattern of incorporation of radioactive glucose into cellulose which indicates that new cellulose is laid down during the graviresponse throughout the pulvinus with no difference in the amount of new cellulose between the top and bottom halves (fig. 9). The increase in activity of glucan synthases I and II

does correspond closely to the time period of maximum bending rate in gravistimulated pulvini (Brock et al. 1989). Steady state cellulose synthesis occurs when the bending process is declining in rate (12–24 h of gravistimulation). Since cell wall synthesis is equal in top and bottom halves of pulvinus tissue, cell wall synthesis cannot be the driving force for the graviresponse but, rather, is a consequence of the elongation process. Thus, we believe that the graviresponse is more likely to be caused by changes in cell wall plasticity and/or osmotic potential. This conclusion is also supported by other investigators (Arslan and Bennett-Clark 1958; Cosgrove 1990; Gibeaut et al. 1990). In addition, we investigated the effect of cellulose synthesis inhibition on the graviresponse with DCBN, a known cellulose synthesis inhibitor (Hogetsu et al. 1974; Montezenos and Delmer 1980). This experiment showed that DCBN-treated pulvini could still gravirespond, producing an average curvature equal to at least 87% of the average curvature of the control pulvini over a 24 h period. These results appear further to support our conclusion that cellulose synthesis is not required for initiation of the graviresponse. However, it must be noted that the efficiency of cellulose synthesis inhibition by DCBN in oat stem tissue has never been quantified, and the efficiency of DCBN uptake with our experimental procedures has also not been demonstrated. Therefore, the cellulose synthesis inhibition results are only tentative. Dayanadan et al. (1982) reported a similar finding with cereal grass stem segments fed a 10 mM solution of DCBN through moistened filter paper. They found that these segments were able to gravirespond with a curvature equal to approximately 40% of the control pulvini over a 48 h period.

One result that is difficult to explain is the finding that glucan synthase activity begins to drop after approximately 10 h of gravistimulation, while cellulose synthesis has not yet reached its highest rate (between 12 and 24 h). The lag between maximum measured glucan synthase activity (around 10 h) and beginning of maximum cellulose synthesis (around 12 h) could result from further processing of a complete cellulose synthase complex (rosette structures [Delmer 1987]) before cellulose synthesis can begin. Also, our cellulose assay will not detect small molecules of cellulose. Small molecules of cellulose would end up in the DMSO-soluble fraction (callose). These two factors could account for the delay between our measured increase in glucan synthase activity and the detection of cellulose synthesis.

The continued rapid rate of cellulose synthesis, even though the glucan synthase activity has dropped back to near control levels, is more difficult to understand, and any explanation is purely speculative. It is possible that a larger pool of glucan synthase molecules is produced early during the graviresponse than will finally end up in the complete cellulose synthase complexes. Therefore, our assay would detect a larger amount of glucan synthase activity in vitro during the early stages of the graviresponse, while later in the response, when the cellulose synthase complexes are complete, there could actually be fewer glucan synthase molecules active in the cell. Therefore, our in vitro assay would detect a decrease in enzyme activity even though cellulose synthesis in vivo is occurring at maximal rate.

In conclusion, we have found that the ultrastructure of cell walls does change during the graviresponse; cell walls from lower halves of graviresponding pulvini become less dense. We also found that cellulose is synthesized equally in top and bottom halves of graviresponding pulvini during the entire graviresponse. There appear to be lag, exponential, steady state, and declining rate phases corresponding to 0–6 h, 6–12 h, 12–24 h, and more than 24 h of gravistimulation. We also determined that glucan synthase I and II activities are equal in top and bottom halves of gravistimulated pulvini, thus indicating that cell wall synthesis is not driving cell elongation in the bottom halves. We believe that increased glucan synthase activity occurs as a result of the graviresponse and is not itself playing a key role in its initiation.

Acknowledgments

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Literature cited

Arslan, N., and T. A. Bennett-Clark. 1958. Geotropic behavior of grass nodes. Exp. Bot. 11:1–12.

Brock, T. G., and P. B. Kaufman. 1990. Movement in grass shoots. Pages 59–71 *in* R. L. Satter, H. Gorton, and T. Vogelmann, eds. The pulvinus: motor organ for leaf movement. Monograph, American Society of Plant Physiologists, Rockville, Md.

Brock, T. G., C. R. Lu, N. S. Ghosheh, and P. B. Kaufman. 1989. Localization and pattern of graviresponse across the pulvinus of barley (*Hordeum vulgare*). Plant Physiol. 91: 744–748.

Carpita, N. C. 1984. Cell wall development in maize coleoptiles. Plant Physiol. 76:205–212.

Cosgrove, D. J. 1990. Gravitropism of cucumber hypocotyls: biophysical mechanism for altered growth. Plant, Cell Envir. 13:235–241.

Dayanadan, P., C. I. Franklin, and P. B. Kaufman. 1982. Linkage between gravity perception and response in the grass leaf-sheath pulvinus. Physiologist 25:101–102.

Dayanadan, P., F. V. Hebard, and P. B. Kaufman. 1976. Cell elongation in the grass pulvinus in response to geotropic stimulation and auxin application. Planta 131:245–252.

Delmer, D. P. 1987. Cellulose biosynthesis. Ann. Rev. Plant Physiol. 38:259–290.

Gibeaut, D. M., N. Karuppiah, S-R. Chang, T. G. Brock, B. Vadlamudi, D. Kim, N. S. Ghosheh, D. L. Rayle, N. C. Carpita, and P. B. Kaufman. 1990. Cell wall and enzyme changes during the graviresponse of the leaf-sheath pulvinus of oat (*Avena sativa*). Plant Physiol. 94:411–416.

Hogetsu, T., H. Shibaoka, and M. Shimokoriyama. 1974. Involvement of cellulose synthesis in actions of gibberellin and kinetin on cell expansion: 2,6-dichlorobenzonitrile as a new cellulose synthesis inhibitor. Plant Cell Physiol. 15: 389–393.

Kato, Y., and D. J. Nevins. 1986. Fine structure of (1 → 3), (1 → 4)-β-D-glucan from *Zea* shoot cell walls. Carbohydr. Res. 147:69–85.

Kaufman, P. B., T. G. Brock, I. Song, Y. B. Rho, and N. S. Ghosheh. 1987. How cereal grass shoots perceive and respond to gravity. Am. J. Bot. 74:1446–1457.

Meier, H., L. Buchs, A. J. Buchala, and T. Homewood. 1981. (1 → 3)-β-D-glucan (callose) is a probable intermediate in biosynthesis of cellulose of cotton fibers. Nature 289:821–822.

Montezenos, D., and D. P. Delmer. 1980. Characterization of inhibitors of cellulose synthesis in cotton fibers. Planta 148:305–311.

Ray, P. M. 1979. Maize coleoptile cellular membranes bearing different types of glucan synthase activity. Pages 135–146 *in* E. Reid, ed. Plant organelles. Horwood, Chichester.

Song, I., C. R. Lu, T. G. Brock, and P. B. Kaufman. 1988. Do starch statoliths act as the gravisensors in cereal grass pulvini? Plant Physiol. 86:1155–1162.

Spurr, A. 1969. A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26: 31–43.

Walton, J. D., and P. M. Ray. 1982. Inhibition by light of growth and Golgi-localized glucan synthase in the maize mesocotyl. Planta 156:302–308.

Woodward, J. R., G. B. Fincher, and B. A. Stone. 1983. Water soluble (1 → 3), (1 → 4)-β-D-glucans from barley (*Hordeum vulgare*) endosperm. II. Fine structure. Carbohydr. Polym. 3:207–225.